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Original Paper

Inhibition of Pgp Activity and Cell Cycle-dependent Chemosensitivity to Doxorubicin in the Multidrug-resistant LoVo Human Colon Cancer Cell Line

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To determine whether the cell cycle affects multidrug resistance (MDR) and its reversal, doxorubicin (DOX) cytotoxicity and the effect of inhibition of P-glycoprotein (Pgp) activity by verapamil (VER) were investigated in MDR LoVo cell lines (LoVo-R) in different phases of the cell cycle. Synchronised cells were obtained by exposing cells for 24 h to non-toxic concentrations (40 nmol/l) of methotrexate (MTX), which induced a reversible blockade in the S phase. DOX cytotoxicity was higher if cells were exposed to DOX shortly after the pretreatment with MTX, when most cells were in the S phase of the cell cycle. At that time, the DOX concentration inhibiting cell growth by 50% (IC_{50}) was decreased by approximately 4-fold compared to non-synchronised cycling cells. DOX cytotoxicity remained high during the transition from the S to the G_2M phase, but was reduced when the cells had shifted to the G_2M phase. Inhibition of Pgp activity by VER (6 μmol/l) enhanced DOX uptake and resulted in an intracellular nuclear compartmentalisation of DOX in LoVo-R cells. These effects were not significantly different (P = NS) in the different phases of the cell cycle. However, similar increases in intracellular DOX uptake due to the inhibitory effect of VER on Pgp greatly potentiated DOX cytotoxicity in LoVo-R cells synchronised in the S or G_2M phase compared with non-synchronised cycling cells. The ratio between DOX IC_{50} in the absence and presence of VER in LoVo-R cells synchronised in the S phase and in cycling cells was 11.1 and 4.1, respectively ($P < 0.01$). This greater potentiation could be explained by the increased chemosensitivity of the S- and G_2M-phase cells to intracellular DOX concentration compared with the non-synchronised cells. Finally, the combination of synchronisation by MTX and of inhibition of Pgp activity by VER produced a considerable reduction in DOX IC_{50} (approximately 50-fold) in LoVo-R cells compared with the cells not treated with MTX and VER. In conclusion, this study demonstrates that, in LoVo-R cells, the effect of Pgp inhibition on DOX cytotoxicity is dependent on cell cycle phase. DOX cytotoxicity is maximal when inhibition of Pgp activity occurs during the S/G_2M phases.

Key words: cell cycle, Pgp, verapamil
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INTRODUCTION

ALTHOUGH EXPERIMENTAL evidence indicates that drug resistance can be overcome *in vitro* in multidrug resistant (MDR) cell lines by treating cells with chemosensitisers, clinical MDR reversal remains an intriguing issue [1–3]. Clinical resistance could depend on several variables that prevent the interaction of the antineoplastic drug with the cellular target site, by interfering with drug transport, drug metabolism, and bioavailability of the intracellular drug target [4, 5]. The efficacy of drug treatment depends on the bioavailability of the intracellular target during drug exposure or on the possibility of the drug reaching the intracellular target in an amount sufficient to exert its cytotoxic effect. In MDR cells, this latter phenomenon is prevented by the membrane P-glycoprotein (Pgp) encoded by the *MDR1* gene. This protein extrudes drugs from the intracellular compartment or results in an impaired intracellular compartmentalisation of anticancer

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drugs, preventing them from reaching the intracellular target site [6–9]. However, inhibition of Pgp activity would not be sufficient to restore drug chemosensitivity if the intracellular drug target is not bioavailable during drug exposure [4, 5] because of qualitative or quantitative modifications, or because cells are in a cell cycle phase in which interaction between drug and intracellular target is ineffective. Some drugs belonging to the MDR spectrum, such as vincristine, are typical phase-specific drugs. Others, such as anthracyclines or epipodophyllotoxins, are more active during certain phases of the cell cycle, though they are not typical phase-specific drugs [10, 11].

Doxorubicin (DOX) is a drug involved in MDR. Although its mechanism of action is not clearly defined, DOX may intercalate into the DNA and/or react with topoisomerase II [12]. As might be expected of compounds that inhibit DNA function, maximal toxicity occurs in the S phase of the cell cycle [11] and/or in the G_2M phase, when concentrations of the 170-kDa topoisomerase isoform are higher, and/or when higher susceptibility to drug-induced DNA cleavage occurs [13].

While the effect of inhibition of Pgp activity on DOX cytotoxicity [12, 14] is well known, little work has been devoted to the study of the potentiation of DOX cytotoxicity in relation to the cell cycle phase in which inhibition of Pgp activity is performed [15]. More studies on this issue could be important for clinical treatments with reversers. Some positive results have been obtained with reversal treatments in haematological neoplasms that generally have rapid growth kinetics, but results have not been as promising in slow proliferating cancers, such as solid tumours [1–3]. It is worth considering the possibility that chemotherapeutic treatment of MDR cells when they are in the most chemosensitive phase of the cell cycle may depend on their growth kinetics [16].

In this paper, we have investigated the effect of Pgp inhibition on DOX cytotoxicity as a function of the cell cycle phase in which this inhibition occurs. We have used the MDR LoVo cell line (LoVo-R) as an experimental model. Cell synchronisation was obtained by treating cells with a non-toxic concentration of methotrexate (MTX), which did not cause any detectable cytotoxicity, as reported previously [17].

MATERIALS AND METHODS

Drugs

DOX was obtained from Pharmacia-Farmitalia (Milan, Italy), MTX was purchased from Lederle (Catania, Italy), and racemic verapamil (VER) from Knoll (Ludwigshafen, Germany). Drugs were dissoved in water under sterile conditions just before use. [^{14}C]-DOX (specific activity 55 mCi/mmol) was obtained from Amersham (Buckinghamshire, U.K.).

Cell lines

LoVo cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The LoVo-R cell line was obtained by selection of the parental cell line (LoVo) with 100 ng/ml DOX, as described previously [18]. LoVo-R cells were maintained in the continued presence of the same DOX concentration for more than 12 months, until 24 h before testing. LoVo-R cells expressed levels of *MDR1* mRNA about 18-fold higher than those of LoVo-sensitive cells, as shown by Nothern and dot blot analyses. We have previously demonstrated that LoVo-sensitive cells also express detectable amounts of *MDR1* mRNA, though at very low levels [18]. Cells were propagated in Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Seralab, Sussex, U.K.), 50 μg/ml streptomycin, and 50 U/ml of penicillin G. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Synchronisation of cell culture and determination of cell synchronisation

Exponentially growing cells were incubated with 40 nmol/l MTX for 24 h under standard culture conditions. After treatment, the drug-containing medium was removed, the cells washed with phosphate-buffered saline (PBS) and fresh medium provided. At different intervals, synchronisation was verified by flow cytometry procedures. The DNA content of the cells was determined by FACScan (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) analysis after staining the nuclei with propidium iodide. The SFIT model was used to calculate the cell cycle phase distribution.

Cytotoxic effects

The cytotoxic effects of the pharmacological treatment were determined by clonogenic assay in liquid medium, as previously described [9]. Drug cytotoxicity was calculated as the percentage of cell survival in drug-treated cultures compared with that in untreated controls. Results are reported as drug concentrations (nmol/l) inhibiting cell clonogenicity by 50% (IC_{50}). IC_{50} was extrapolated by regression analysis of the experimental data. Cells not treated with the drugs were used as controls. In the experiments with VER (6 μmol/l), cells treated with VER alone were used as controls. In synchronised cells, treatment with DOX for 1.5 h in the presence or absence of VER was performed at different times of recovery after pretreatment with MTX. The concentrations of VER alone did not cause any cytotoxic effect on non-synchronised or on MTX-pretreated LoVo and LoVo-R cells. Colony efficiency in MTX synchronised cells was not significantly different from the controls.

DOX uptake and subcellular location

Exponentially growing and synchronised cells, $3–10 \times 10^6$ in 10 ml of medium, seeded in 90-mm Petri dishes (Falcon, New Jersey, U.S.A.), were incubated for 1.5 h at 37°C with 15 ml of fresh medium containing appropriate concentrations of [^{14}C]-DOX, with or without 6 μmol/l VER. Radioactive medium was subsequently withdrawn and Petri dishes chilled on ice and quickly washed three times with ice-cold PBS. Cells were harvested by treatment with trypsin and counted with a haemocytometer. [^{14}C]-DOX uptake was determined by liquid scinitillation counting. Results (expressed as pmol DOX/10^6 cells) are reported as intracellular DOX content; where specified, intracellular DOX content was corrected for the variation in cell volume (determined by a Coulter Mod ZM apparatus) that occurred after MTX treatment. The cell volume of non-synchronised cells was used as the reference value (see Table 1).

DOX subcellular distribution was visualised with a Nikon, Diaphot-TMD fluorescence microscope (Nikon, Japan) equipped with a high pressure HBO 100 W DC mercury lamp, appropriate filters, a C2400-87 intensified CCD Hamamatsu camera (Hamamatsu, Japan), and a custom-made microscope image analysis system (TGA, Italy). For quantification of the nuclear/cytoplasmic ratio (N/C ratio) for DOX,

Table 1. Cytotoxicity of doxorubicin (DOX) and effect of verapamil (VER) treatment in LoVo-R cells in different phases of the cell cycle

Recovery time† (h)	Cells in different cell cycle phases (%)			Cell volume ratio‡	DOX IC_{50} (μmol/l)				VER enhancement‡‡
	G_1	S	G_2/M		−VER§		+VER‖		
Control	46.8±9.2	35.8±6.8	17.4±2.4	1	34.03±5.07	(1.0)¶	8.31±2.54	(1.0)††	4.1
0	4.5±2.1	91.0±2.6	4.5±4.0	1.2	7.44±1.31	(4.6)**	0.67±0.22	(12.4)**	11.1**
1	3.8±1.7	90.8±3.8	5.0±2.9	1.3	8.37±0.90	(4.1)**	0.80±0.10	(10.4)**	10.5**
2	4.5±2.1	86.5±2.6	9.0±2.6	1.3	7.87±2.02	(4.3)**	0.92±0.22	(9.0)**	8.6**
4	7.5±3.5	73.3±6.7	19.3±5.3	1.3	9.01±1.88	(3.8)**	0.97±0.13	(8.6)**	9.3**
6	13.0±7.32	27.3±8.5	64.0±8.4	1.6	12.06±0.94	(2.8)**	1.38±0.08	(6.0)**	8.7**
8	28.5±6.1	20.8±8.8	50.8±6.2	1.6	19.26±3.85	(1.8)**	2.19±0.59	(3.8)**	8.8**
12	17.8±6.9	39.8±10.2	42.5±10.4	1.4	23.52±2.43	(1.4)*	3.30±0.37	(2.5)**	7.1**
16	45.0±4.1	27.3±6.9	32.7±2.6	0.8	35.69±8.03	(1.0)	6.89±0.66	(1.2)	5.2
24	44.3±4.1	39.8±3.1	16.0±5.5	1.1	38.35±9.09	(0.9)	9.15±2.48	(0.9)	4.2
48	39.8±6.02	44.0±7.4	16.3±7.6	1.0	38.49±7.45	(0.9)	9.67±1.63	(0.9)	4.0

†Times of recovery after synchronisation with 40 nmol/l MTX for 24 h. Control cells were not treated with MTX.‡ Ratios between cell volume at different recovery times and cell volume of the control cells. §DOX IC_{50} in the absence or ‖presence of 6 μmol/l VER. Cells were exposed to DOX with or without VER for 1.5 h. ¶,††Ratios between DOX IC_{50} of control cells not treated with MTX, and DOX IC_{50} of cells at the defined recovery time in absence (−VER) or presence (+VER). ‡‡Ratios between § and ‖. *,**P significantly different compared with the control cells; mean ± S.D. of at least four experiments. IC_{50}, concentration inhibiting cell growth by 50%.

cells grown on chamber slides were incubated for 1.5 h with 6 μmol/l DOX in the presence or absence of 6 μmol/l VER. Microscopic observation was performed with the microscopic slide kept at 37°C. Experimental data are the means of 30–50 cell measurements for each treatment.

Statistics

For statistical analysis the t-test was used. Results were considered statistically significant at $P \leq 0.05$.

RESULTS

DOX cytotoxicity as a function of cell cycle phase and effect of VER treatment

Treatment of LoVo-R cells with 40 nmol/l of MTX for 24 h caused a remarkable enrichment of S phase cells, whereas most cells were in the G_2M phase following 6 h of recovery after drug removal. The synchronising effect of MTX was reversible, and 24–48 h after MTX treatment the percentage of cells in the $G_{0/1}$, S and G_2M phases was approximately the same as in the untreated controls (Figure 1, Table 1). Forty nanomoles/litre of MTX did not affect the colony formation of LoVo and LoVo-R cells. The chemosensitivity of LoVo and LoVo-R cells to MTX was approximately the same. After 24 h of treatment, the MTX IC_{50} (mean ± S.D.) was 121.3 ± 27.3 nmol/l and 134.1 ± 46.6 nmol/l in LoVo and LoVo-R cells, respectively.

Table 1 shows DOX IC_{50} values at different times after treatment of LoVo-R cells with 40 nmol/l MTX for 24 h. Cells were exposed to DOX for 1.5 h. DOX IC_{50} was lower if cells were exposed to the anthracycline shortly after treatment with MTX, when most cells were synchronised in the S phase. The cytotoxic effect of DOX remained high during the transition from the S to the G_2M phase, but was reduced when cells shifted to the G_2M phase.

Inhibition of Pgp activity by 6 μmol/l VER greatly enhanced the cytotoxic effect of DOX in LoVo-R cells. This enhancement was higher in the cells synchronised in the S and G_2M phases. DOX IC_{50} was decreased by approximately 10-fold when more than 90% of LoVo-R cells were in the S phase of the cell cycle, whereas DOX IC_{50} was decreased by approximately 4-fold in cells not synchronised by MTX pretreatment ($P < 0.01$) (Table 1). In LoVo-sensitive cells, a significant ($P < 0.01$) potentiation of DOX cytotoxicity was achieved only if inhibition of Pgp activity by VER was performed when cells were in the S or G_2M phases of the cell cycle. The DOX IC_{50}, in the presence of 6 μmol/l VER was decreased ($P < 0.01$) 2-fold in LoVo cells synchronised in the S phase and by 1.3 (P = NS) in non-synchronised cells (Table 2).

Synchronisation by MTX and inhibition of Pgp activity by VER dramatically increased DOX cytotoxicity in LoVo-R cells. DOX IC_{50} in non-synchronised LoVo-R cells was 34.03 ± 5.07 μmol/l, whereas the DOX IC_{50} in LoVo-R cells synchronised in the S phase and treated with VER was 0.67 ± 0.22 μmol/l (ratio = 50.8). In LoVo-sensitive cells, the effect of synchronisation in the S phase plus inhibition of Pgp activity by VER increased DOX cytotoxicity 7.6-fold.

DOX uptake and compartmentalisation in LoVo-sensitive and LoVo-R cells as a function of cell cycle phase and effect of VER

Cellular DOX content was investigated in sensitive and resistant cells during the recovery time from treatment with non-cytotoxic concentrations of MTX (40 nmol/l). After 1.5 h of exposure to 1 μmol/l of DOX, the intracellular DOX content in LoVo-R cells was approximately 3-fold lower than in LoVo cells. Maximal intracellular DOX content in both LoVo and LoVo-R cells was observed after 6–8 h of recovery, when the majority of the cells were in the G_2M phase of the cell cycle (Figure 2). This increase in intracellular DOX content was not associated with an increment of intracellular DOX concentration because of a variation in cell volume during the different phases of the cell cycle. The median volume was 1068 ± 91 fl and 1091 ± 165 fl in non-synchronised LoVo and LoVo-R cells, respectively, whereas it was 1706 ± 92 fl and 1746 ± 36 fl in LoVo and LoVo-R cells synchronised in the G_2M phase, respectively. VER (6 μmol/l) enhanced cellular uptake of DOX in LoVo-R cells, but this increase was nearly identical in all of the different phases of the cell cycle (approximately 2.5-fold) (Figure 2). In addition, VER treat-

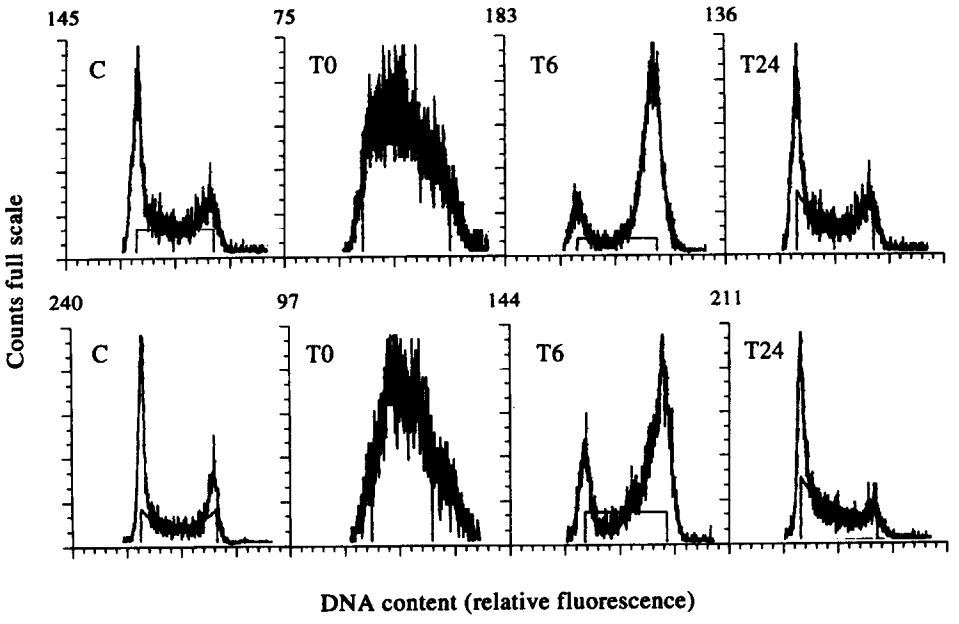

Figure 1. Flow cytometric analysis of LoVo (upper) and LoVo-R cells (lower) after 24 h of exposure to 40 nmol/l methotrexate (MTX). C, control cells not treated with MTX; T0, cells exposed to MTX for 24 h. Cells were treated with MTX, washed, incubated in drug-free medium, and analysed 6 h (T6) and 24 h (T24) later.

Table 2. Cytotoxicity of doxorubicin (DOX) and effect of verapamil (VER) treatment in LoVo-sensitive cells in different phases of the cell cycle

Recovery time† (h)	Cells in different cell cycle phases (%)			Cell volume ratio‡	DOX IC_{50} (μmol/l)				VER enhancement‡‡
	$G_{0/1}$	S	G_2/M		−VER§		+VER‖		
Control	41.5±5.5	43.8±5.6	14.7±3.8	1	0.61±0.08	(1.0)¶	0.46±0.06	(1.0)††	1.3
0	3.7±1.3	92.9±2.3	3.4±1.5	1.3	0.16±0.03	(3.8)**	0.08±0.01	(5.8)**	2.0**
6	15.0±5.7	26.8±8.8	58.2±9.3	1.6	0.24±0.01	(2.5)**	0.14±0.03	(3.3)**	1.7*
24	43.9±6.7	38.6±4.2	16.2±3.2	1.1	0.58±0.04	(1.1)	0.49±0.06	(0.9)	1.2

For abbreviations see legend to Table 1.

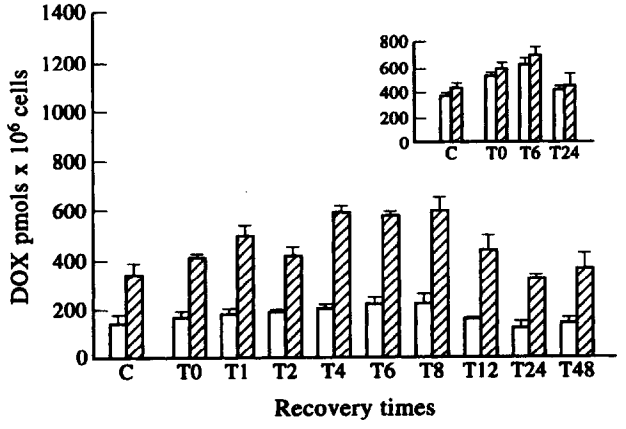

Figure 2. Effect of synchronisation by methotrexate (MTX) on the intracellular doxorubicin (DOX) content in the presence (▨) or absence (□) of verapamil (VER) in LoVo-R cells. Cells were exposed for 1.5 h to 3 μmol/l DOX ± 6 μmol/l VER at different recovery times after MTX pretreatment. C, control cells not treated with MTX. Inset: DOX uptake in LoVo-sensitive cells. Data obtained from at least four experiments. Bars, standard deviation.

ment did not result in any variation in the cell volume of LoVo and LoVo-R cells (data not shown).

LoVo-R cells exposed to 3 μmol/l DOX for 1.5 h showed predominantly intracytoplasmic drug accumulation. Intracytoplasmic DOX compartmentalisation was not observed in drug-sensitive LoVo cells, where DOX was localised mainly in the nucleus. The N/C DOX fluorescence ratio was 0.65 ± 0.02 in LoVo-R and 2.1 ± 0.1 in LoVo cells, without significant differences (P = NS) between synchronised and non-synchronised cells (Table 3). Inhibition of Pgp activity by 6 μmol/l VER allowed DOX to be redistributed in the nucleus of LoVo-R cells. VER affected the DOX N/C ratio of S-phase cells as much as that of non-synchronised cells (Table 3).

Cytotoxic effect of intracellular DOX in LoVo-R cells

To ascertain whether MDR cells have a different chemosensitivity to intracellular DOX content in the different phases of the cell cycle and to determine the effect of VER, LoVo-R cells synchronised in the S phase (recovery time from MTX pretreatment = 0) and non-synchronised cycling cells were exposed for 1.5 h to various extracellular DOX concentrations. The intracellular DOX content corrected for cell volume (see Materials and Methods) was determined at the

Table 3. Subcellular doxorubicin (DOX) distribution in LoVo-sensitive (LoVo) and LoVo-resistant (LoVo-R) cells

Recovery time	N/C ratio*			
	LoVo		LoVo-R	
	-VER	+VER	-VER	+VER
Control	2.10 ± 0.10	2.64 ± 0.58	0.65 ± 0.02	1.4 ± 0.02
0	2.47 ±0.51	2.67 ± 0.90	0.53 ± 0.12	1.6 ± 0.47
24	3.01 ±0.94	2.54 ± 1.01	0.71 ± 0.21	1.9 ± 0.23

*Nucleus/cytoplasmic fluorescence ratio. Cells were exposed to 3 μmol/l DOX in the presence or absence of 6 μmol/l VER.

same extracellular DOX concentrations used in the clonogenic assay (Figure 3).

LoVo-R cells in the S phase of the cell cycle were much more chemosensitive to intracellular DOX than non-synchronised cells (Figure 3). The intracellular DOX content inhibiting cell growth by 50% (DOX IC_{50int}), as determined by regression analysis of the dose–response curves reported in Figure 3, was 546 ± 56 pmol DOX × 10^6 cells and 1782 ± 371 pmol DOX × 10^6 cells in S-phase and non-synchronised LoVo-R cells, respectively. Exposure to 6 μmol/l VER increased the efficacy of intracellular DOX content in synchronised and non-synchronised cells, probably as a consequence of the intracellular DOX redistribution. However, S-phase cells were more chemosensitive to intracellular DOX than non-synchronised LoVo-R cells, even in the presence of VER; the DOX IC_{50int} was 192 ± 38 pmol DOX × 10^6 cells and 834 ± 241 pmol DOX × 10^6 cells, respectively, in the S-phase and non-synchronised LoVo-R cells (Figure 3).

DISCUSSION

It is well known that tumours with rapid growth kinetics are more sensitive to antineoplastic agents than slow proliferating tumours. In addition, many clinical trials have demonstrated that the use of chemosensitisers in the clinic could be efficacious only in tumours expressing Pgp and with rapid growth kinetics. Conversely, reversal treatments in slow proliferating cancers are generally ineffective and could be associated with increased toxicity in tissues expressing Pgp and with a short doubling time, such as colon mucosa or haemopoietic stem cells [1–3]. Although many biochemical mechanisms could account for the efficacy in fast cycling cells, it must be considered that many antineoplastic drugs related to MDR are more active in the S or G_2M phases of the cell cycle. In the reversal treatment of slow proliferating human tumours, the fraction of tumour cells exposed to the antineoplastic drug and chemosensitiser when they are in the most chemosensitive phase of the cell cycle is extremely low. This raises the question of whether inhibition of Pgp activity performed at different stages of the cell cycle can determine different cytotoxic effects, or whether the differences in cytotoxicity are simply due to variations in the chemosensitivity of the cells in the different cell cycle phases.

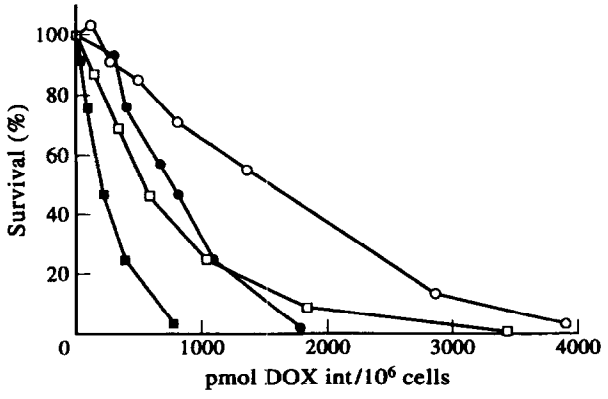

Figure 3. Cell survival as a function of intracellular doxorubicin (DOX) content corrected for cell volume variation (see Materials and Methods) in S-phase synchronised (□■) and non-synchronised (○●) LoVo-R cells, without verapamil (VER) (open symbols) and in the presence of 6 μmol/l VER (solid symbols). Each data point represents the average of at least four independent experiments. The relative standard deviation was <30%.

In agreement with previous data [10], we found that LoVo-R cell lines synchronised in the S phase by pretreatment with non-toxic concentrations of MTX are approximately 4- to 5-fold more sensitive to subsequent exposure to DOX than non-synchronised cells. The cytotoxic effect of DOX remained high during the transition from the S to the G_2M phase, but was reduced when cells shifted to the G_2M phase.

In LoVo-sensitive cells, MTX synchronisation had the same effects as in the resistant cells, thus indicating that MTX synchronisation did not affect Pgp-mediated resistance.

Inhibition of Pgp activity by VER greatly enhanced the cytotoxic effect of DOX in LoVo-R cells. This enhancement was higher in the cells synchronised in the S and G_2M phases (range from 7.1- to 11.1-fold) than in cells not synchronised by MTX pretreatment (approximately 4-fold, $P < 0.01$). Therefore, the sensitisation factor by VER was 2- to 3-fold higher in synchronised cells than in the non-synchronised counterparts.

A significant ($P < 0.05$) potentiation (approximately 2-fold) of DOX cytotoxicity by VER was achieved in LoVo-sensitive cells only if inhibition of Pgp activity by VER occurred when cells were in the S and G_2M phases of the cell cycle (in non-synchronised LoVo-sensitive control cells potentiation of DOX cytotoxicity was 1.3-fold (NS)). It should be noted that LoVo-sensitive cell lines constitutively express *MDR1* mRNA even if at very low levels compared with LoVo-R cells [19]. Our data therefore suggest that inhibition of Pgp activity affects DOX cytotoxicity in MDR cells expressing low levels of *MDR1* mRNA only when cells are in the most chemosensitive phase of the cell cycle. Some studies have indicated that the level of *MDR1* mRNA expression in human tumours is lower than in MDR cell lines obtained by drug selection [2], and we previously demonstrated that the level of *MDR1* mRNA expression in LoVo-sensitive cell lines is superimposable on that observed in human colon carcinomas [18].

Pgp determines reduced intracellular drug accumulation [6] and/or altered intracellular drug compartmentalisation, which prevent the drug from reaching the intracellular target sites. DOX is localised mainly in the nucleus of sensitive cells, but has a preferential cytoplasmic distribution in MDR cells. Inhibition of Pgp activity results in a nuclear DOX redistribution of MDR cells [7–9]. We found that Pgp inhibition by VER has similar effects on DOX uptake in synchronised and non-synchronised LoVo-R cells. In agreement with the data reported by Tarasiuk and associates [15], VER increased intracellular DOX concentration approximately 2.5-fold both

in synchronised and non-synchronised cells. Moreover, even if the determination of the DOX N/C ratio was inaccurate, particularly as quenching of nuclear fluorescence could occur if nuclear anthracyclines are intercalated into the DNA strands, we found that the effects of VER on DOX N/C ratio were similar in the synchronised and non-synchronised cells. These data indicate that the increased potentiation of DOX cytotoxicity observed in synchronised cells as a consequence of Pgp inhibition by VER could not be ascribed to the differential effects of Pgp inhibition on intracellular DOX pharmacokinetics.

In LoVo-sensitive cells, only slight increases in cellular DOX content were observed after VER exposure, independent of the cell cycle phase in which VER plus DOX treatment was performed. These differences were not significant because of large standard deviations (P = NS).

Comparison of intracellular DOX concentration with DOX cytotoxicity in LoVo-R cells indicated that, both in the cells treated with VER and in synchronised cells, lower concentrations of DOX were required to achieve the same cytotoxic effects as in the control cells.Thus, synchronised cells and resistant cells in which Pgp activity is inhibited are more sensitive to intracellular DOX concentrations. For equitoxic effects, S- or G_2M-phase cells required lower intracellular drug concentrations than non-synchronised cells, most of which were in the G_1 phase. This probably occurs because, in the synchronised cells, the intracellular target(s) is more bioavailable for interaction with DOX. The latter interacts with topoisomerase II which, as recently reported by Kimura and associates, undergoes qualitative and quantitative modifications during the S and G_2M phases of the cell cycle [10]. Moreover, the redistribution of drug due to Pgp inhibition increases the drug concentration at the intracellular target site, resulting in an increased sensitivity to the intracellular drug concentrations [8, 19]. It is worth considering that the curves obtained by plotting the intracellular DOX concentrations and the percentage of cell survival (as reported in Figure 3) had different slopes in synchronised and non-synchronised cells. Similar increases in the intracellular DOX concentration due to the inhibition of Pgp resulted in higher cytotoxic effects in the synchronised cells compared with the non-synchronised MDR cells. According to this, in LoVo-R cells in which the activity of Pgp was inhibited by VER, the ratio between DOX IC_{50} in non-synchronised cells and in cells synchronised in the S or G_2M phases was greater than the ratio between DOX IC_{50} in non-synchronised and synchronised LoVo-R cells not exposed to VER, confirming that VER increased DOX efficacy in synchronised cells compared with non-synchronised cells.

Finally, the combined effect of synchronisation and inhibition of Pgp activity dramatically increased the intracellular drug sensitivity to DOX, probably because of the cooperation of the two mechanisms, i.e. an increased drug concentration at the intracellular target site, due to drug recompartmentalisation, occurs when the intracellular target site is more bioavailable for interaction with the antineoplastic drug, as a consequence of synchronisation. In S-phase LoVo-R cells treated with VER, the DOX IC_{50int} was 192 ± 38 pmol DOX $\times 10^6$ cells, whereas in the cells not treated with MTX and VER, the DOX IC_{50int} was 1782 ± 371 pmol DOX $\times 10^6$ cells. Because of this dramatic increase in the intracellular DOX efficacy, the extracellular DOX concentration for equitoxic effects was decreased by approximately 50-fold in LoVo-R cells treated with MTX and VER compared with untreated control cells.

In conclusion, this study demonstrates that, in LoVo-R cells, the efficacy of treatment with DOX plus an inhibitor of Pgp activity is schedule dependent. For maximal efficacy of the reversal teatment, cells must be treated when they are in the most chemosensitive phase of the cell cycle. Increased DOX cytotoxicity appears to be related to the differential chemosensitivity of LoVo-R cells to intracellular DOX concentration in the different phases of the cell cycle. In contrast, inhibition of Pgp activity has the same effect on drug transport and intracellular compartmentalisation in all phases of the cell cycle.

Although *in vivo* extrapolations to human tumours from *in vitro* experimental data would not be appropriate, the data reported here are consistent with the results of clinical trials using chemosensitisers in MDR neoplasms with fast growth kinetics, such as haematological neoplasms. It should be noted that, in these tumours, the number of cells in a chemosensitive phase of the cell cycle during reversal treatment is high, whereas in MDR tumours with slow growth kinetics, the fraction of cells sensitive to the combination drug reverser may be extremely low. The inhibition of Pgp activity by reversers could potentiate the cytotoxic effect of the antineoplastic drugs only on normal tissues with higher growth kinetics, without affecting MDR tumour cells. The results of the present work seem to indicate that reversal treatment is useful in a restricted number of MDR solid tumours with fast growth kinetics, but at the same time suggest a reconsideration, in terms of schedule, of the reversal treatments and clinical protocols in use for MDR tumours with slow growth kinetics.

1. Raderer M, Scheithauer W. Clinical trials of agents that reverse multidrug resistance. *Cancer* 1993, **72**, 3553–3563.
2. Dalton WS. Drug resistance modulation in the laboratory and the clinic. *Semin Oncol* 1993, **20**, 64–69.
3. Lehnert M. Reversal of P-glycoprotein-associated multidrug resistance: the challenge continues. *Eur J Cancer* 1993, **29A**, 636–638.
4. Cazin JL, Gosselin P, Cappelaere P, Robert J, Demaille A. Drug resistance in oncology: from concepts to applications. *J Cancer Res Clin Oncol* 1992, **119**, 76–86.
5. De Vita VT. The problem of resistance keynote address. In Mihich E, ed. *Drug Resistance: Mechanisms and Reversal.* Roma, John Libbey CIC, 1990, 7–27.
6. Skovsgaard T. Mechanism of cross-resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* 1978, **38**, 4722–4727.
7. Gervasoni JE Jr, Fields SZ, Krishna S, *et al.* Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy. *Cancer Res* 1991, **51**, 4955–4963.
8. Schuurhuis GJ, van Heijningen THM, Cervantes A, *et al.* Changes in subcellular doxorubicin distribution and cellular accumulation alone can largely account for doxorubicin resistance in SW-1573 lung cancer and MCF-7 breast cancer multidrug resistant tumour cells. *Br J Cancer* 1993, **68**, 898–908.
9. Boiocchi M, Toffoli G. Mechanisms of multidrug resistance in human tumor cell lines and complete reversion of cellular resistance. *Eur J Cancer* 1992, **28A**, 1099–1105.
10. Lee FJF, Flannery DJ, Siemann DW. Modulation of the cell cycle-dependent cytotoxicity of adriamycin and 4-hydroperoxycyclophosphamide by novobiocin, an inhibitor of mammalian topoisomerase II. *Cancer Res* 1992, **52**, 3515–3520.
11. Calabresi P, Parks RE Jr. Antiproliferative agents and drugs used for immunosuppression. In Gilman AG, Goodman LS, Rall TW, Murad F, eds. *The Pharmacological Basis of Therapeutics.* New York, MacMillan, 1985, 1283–1285.

12. Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy. Focus on drug resistance. *Drugs* 1994, **47**, 223–258.
13. Kimura K, Saijo M, Ui M, Enomoto T. Growth state- and cell cycle-dependent fluctuation in the expression of two forms of DNA topoisomerase II and possible specific modification of the higher molecular weight form in the M phase. *J Biol Chem* 1994, **269**, 1173–1176.
14. Toffoli G, Viel A, Tumiotto L, Biscontin G, Rossi C, Boiocchi M. Pleiotropic-resistance phenotype in a multifactorial phenomenon in human colon carcinoma cell lines. *Br J Cancer* 1991, **63**, 51–56.
15. Tarasiuk J, Foucrier J, Garnier-Suillerot A. Cell cycle dependent uptake and release of anthracycline by drug-resistant and drug-sensitive human leukaemic K562 cells. *Biochem Pharmacol* 1993, **45**, 1801–1808.
16. Lee WP. The role of reduced growth rate in the development of drug resistance of HOB1 lymphoma cells to vincristine. *Cancer Lett* 1993, **73**, 105–111.
17. Erba E, Sen S, Lorico A, D'Incalci M. Potentiation of etoposide cytotoxicity against a human ovarian cancer cell line by pretreatment with non-toxic concentrations of methotrexate or aphidicolin. *Eur J Cancer* 1992, **28**, 66–71.
18. Toffoli G, Viel A, Tumiotto L, Maestro R, Biscontin G, Boiocchi M. Expression of the mdr1 gene in human colorectal carcinomas: relationship with multidrug resistance inferred from analysis of human colorectal carcinoma cell lines. *Cancer Chemother Pharmacol* 1992, **29**, 283–289.
19. Toffoli G, Simone F, Gigante M, Boiocchi M. Comparison of mechanisms responsible for resistance to idarubicin and daunorubicin in multidrug resistant LoVo cell lines. *Biochem Pharmacol* 1994, **48**, 1871–1881.

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